

# The combination of conjugated equine estrogens with bazedoxifene prevents streptozotocin-induced diabetes in female mice

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📍 **Disciplines**  
Endocrinology

🔍 **Keywords**  
Bazedoxifene  
Diabetes Mellitus  
Estrogens  
Hormone Replacement Therapy  
Insulin-Secreting Cells

🏠 **Type of Observation**  
Standalone

🔗 **Type of Link**  
Standard Data

🕒 **Submitted** May 13, 2016  
📅 **Published** Jun 6, 2016



**Triple Blind Peer Review**  
The handling editor, the reviewers, and the authors are all blinded during the review process.



**Full Open Access**  
Supported by the Velux Foundation, the University of Zurich, and the EPFL School of Life Sciences.



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## Abstract

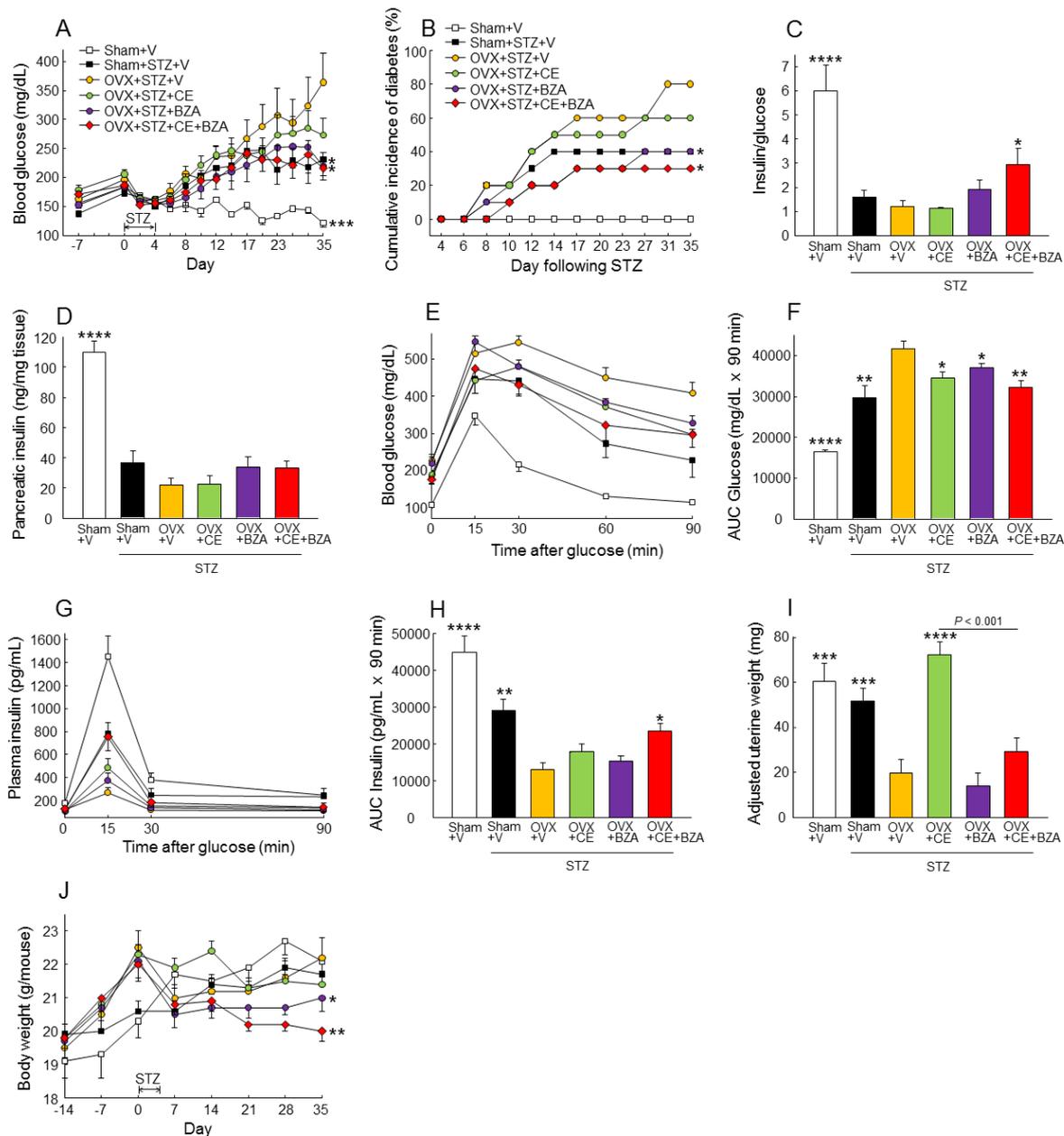
The prevention of streptozotocin (STZ)-induced insulin-deficient diabetes in mice is considered a marker of estrogen mimetic activity in  $\beta$ -cells. The effect of the selective estrogen receptor modulator, bazedoxifene (BZA), alone or combined to conjugated equine estrogens (CE) on the prevention of streptozotocin-induced diabetes, is unknown. Here, we investigated the effects of CE (2.5 mg/kg/d), BZA (3 mg/kg/d) and their combinations on the prevention of STZ-induced diabetes. The combination CE/BZA, BZA alone, and CE alone to a lesser extent, all reduced the incidence and severity of STZ-induced diabetes. These findings demonstrate that in female mice, BZA, alone or combined to CE, acts as an estrogen mimetic in preventing STZ-induced diabetes.

## Introduction

The female hormone  $17\beta$ -estradiol (E<sub>2</sub>) protects pancreatic islet survival from multiple pro-apoptotic insults [1] [2]. Streptozotocin (STZ) induces an increase in islet reactive oxygen species (ROS) as can be encountered following exposure to hyperglycemia or cytokines in diabetes [3] [4]. In mice, E<sub>2</sub> prevents STZ-induced pancreatic  $\beta$ -cell apoptosis predominantly via activation of the estrogen receptor (ER) $\alpha$  in islet  $\beta$ -cells [5]. Therefore, the prevention of STZ-induced insulin-deficient diabetes in mice is considered as a physiological evidence of ER agonistic activity in  $\beta$ -cells [5]. Selective estrogen receptor modulators (SERMs) are compounds that exert tissue-selective ER agonist or antagonist activity. For example, the SERM tamoxifen is an ER $\alpha$  antagonist in  $\beta$ -cells and opposes the beneficial effect of E<sub>2</sub> in islet survival and the prevention of STZ-induced diabetes [5]. A novel approach to menopausal hormone therapy consists in pairing conjugated equine estrogens (CE) with the SERM bazedoxifene (BZA) [6]. The goals of the approach is to provide the benefits of CE by treating hot flashes and preventing menopausal osteoporosis while at the same time protecting the endometrium and breast from CE stimulation with BZA [7]. Using a mouse model of postmenopausal metabolic syndrome, Kim et al. reported that TSEC and BZA alone prevent E<sub>2</sub> deficiency-induced obesity, type 2 diabetes and fatty liver as efficiently as CE and E<sub>2</sub> alone, and importantly, without causing endometrial hyperplasia [8] [9]. The effect of the combination CE/BZA and the effect of BZA alone on the prevention of STZ-induced diabetes are unknown.

## Objective

We investigated the preventive effects of CE (2.5 mg/kg/d), BZA (3 mg/kg/d) and their combinations on the prevention of multiple low-dose STZ-induced diabetes in female mice. We used ovariectomized (OVX) mice as a model of postmenopausal E<sub>2</sub> deficiency.



a

### Figure Legend

**Figure 1.** Mice were subjected to sham or OVX surgeries and were treated as indicated for 5 weeks. Diabetes was induced by multiple (for 5 consecutive days) low-dose (50 mg/kg) intraperitoneal injection of STZ.

(A) Fed blood glucose.

(B) Cumulative incidence of diabetes was calculated as a percentage of hyperglycemic mice (glucose level  $\geq 200$  mg/dL) at each time point.

(C) The ratio of non-fasting insulin (pg/mL) and glucose (mg/dL) at day 35 was used as an index of insulin deficiency in mice.

(D) Pancreatic insulin content.

(E-H) (E) Glucose concentrations during OGTT, (F) area under the curve (AUC) for glucose for (E), (G) insulin concentrations during OGTT and (H) AUC for insulin for (G).

(I) Uterine weights. Means are adjusted for the final body mass as a covariate using the

ANCOVA analysis.

(J) Body weights. Values represent means  $\pm$  SEM (n =6 for sham and n =10 for OVX). Significance is calculated compared to the OVX+STZ treated with vehicle (V) group (\*P <0.05, \*\*P <0.01, \*\*\*P <0.001, \*\*\*\*P <0.0001).

#### **Animals and surgery**

8 week old female C57BL/6J mice (Jackson Laboratory, Bar Harbor, Maine) were housed with a 12 h light-dark cycle. The mice were subjected to either a bilateral OVX or a sham operation under anesthesia with 1.2% avertin solution (i.p.). After a recovery period of 2 weeks, mice were divided into 6 treatment groups as follows: 1) sham+vehicle, 2) sham+STZ+vehicle, 3) OVX+STZ+vehicle, 4) OVX+STZ+CE, 5) OVX+STZ+BZA, 6) OVX+STZ+CE+BZA. Diabetes was induced with 50 mg/kg STZ (dissolved in 50 mM citrate buffer, pH 4.5) or with citrate buffer alone injected intraperitoneally for 5 consecutive days. All compounds were administered orally by gavage to mice once daily for 5 weeks with vehicle (saline, 2% Tween 80, 0.5% methylcellulose), CE 2.5 mg/kg, BZA 3 mg/kg or CE 2.5 mg/kg +BZA 3 mg/kg in the vehicle solution. The administered dosages of CE and BZA were chosen to ensure optimal maintenance of the estrogen action in the absence of uterine growth [15] [16]. All mice received phytoestrogen-free HFD (TD04059, 52% Kcal from anhydrous milk fat, Harlan Teklad, Madison, WI, USA) and water ad libitum during the experimental period. At the end of the study, mice were euthanized by an overdose of avertin, and blood was collected by cardiac puncture. All animal work was performed in compliance with the Institutional Animal Care and Use Committee of Northwestern University and in accordance to NIH guidelines.

#### **Glucose and insulin measurements**

Random-fed blood glucose was measured from blood obtained from the tail vein using a OneTouch Ultra 2 glucose meter (LifeScan, Inc., Milpitas, CA). Hyperglycemia was defined as a non-fasting blood glucose level  $\geq$ 200 mg/dL. The cumulative incidence of diabetes was calculated as a percentage of hyperglycemic mice at each time point. Oral glucose tolerance test (OGTT) was performed at 3 weeks after STZ treatment. Mice were fasted overnight (16 h), and a glucose load (2 g/kg) was administered orally. Blood glucose and plasma insulin levels were measured from the tail vein at 15, 30, 60 and 90 min after administration of glucose. The area under the curve (AUC) for glucose and insulin was calculated for each group of animals during OGTT. Following euthanasia, plasma was separated by centrifugation at 3000 g for 20 min at 4°C and used for the determination of insulin levels using an ELISA kit (Millipore). The pancreas was isolated, homogenized in acidified ethanol, extracted overnight at 4°C, and centrifuged. The insulin content of the supernatant was determined using an ELISA kit (Millipore) and expressed in ng/mg pancreas.

#### **Statistical analyses**

Data were analyzed by one-way ANOVA using SAS software for Windows release 9.2 (SAS Institute Inc., Cary, NC, USA) on the W32\_VSHOME platform. To test for differences in uterine weights among the treatment groups, analysis of covariance (ANCOVA) with final body mass as a covariate was used. Homogeneity of regression assumptions of the ANCOVA model were tested and met in each analysis. Differences in cumulative incidence of diabetes were determined by the log-rank test. The Least Squares Means option using a Tukey–Kramer adjustment was used for multiple comparisons among the treatment groups. Data were presented as the mean  $\pm$  SEM. P values <0.05 were considered statistically significant. **Results & Discussion**

STZ treatment induced a progressive hyperglycemia with a corresponding increase in diabetes incidence, and these changes were more pronounced in OVX mice than in sham-operated mice (Fig. 1A and B). Treatment of OVX mice with BZA or the combination CE/BZA significantly reduced hyperglycemia and decreased the incidence of STZ-induced diabetes at day 35 (Fig. 1A and B). There was a nonsignificant reduction in hyperglycemia and diabetes incidence following treatment with CE alone (Fig. 1A and B). The index of insulin deficiency (insulin/glucose ratio) was less pronounced in the OVX group treated with CE+BZA compared to vehicle, but did not reach significance

following treatment with BZA (Fig. 1C). We did not observe any significant changes in pancreatic insulin content in OVX mice after any of the hormonal treatments, although pancreatic insulin content tended to be higher in mice treated with BZA and CE/BZA (Fig. 1D). Following an oral glucose load, all hormonal treatments improved glucose tolerance in OVX mice (Fig. 1E and F), with mice treated with CE/BZA showing significantly higher glucose-stimulated insulin secretion compared to vehicle-treated mice (Fig. 1G and H). Consistent with previous findings (8), BZA alone did not induce uterine growth (as assessed by increased tissue weight), and prevented uterine growth when combined with CE (Fig. 1I). The combination CE/BZA produced a stronger effect on body weight suppression than other hormonal treatments (Fig. 1J).

Menopause is associated with an increased risk of type 2 diabetes and large randomized controlled trials have conclusively showed that menopausal hormone therapy using CE alone or in combination with a progestogen decreases the risk of type 2 diabetes [10] [11] [12] [13]. The SERM tamoxifen acts as an ER antagonist in  $\beta$ -cells [5]. In fact, tamoxifen therapy in a case-control study of breast cancer survivor was associated with a 24% increased risk of developing diabetes [14]. Therefore, it was critical to determine whether BZA and the combination CE/BZA, which is currently approved by the FDA for menopausal therapy, have beneficial action in  $\beta$ -cells. Using the paradigm of STZ-induced insulin-deficient diabetes in OVX female mice as readout of ER agonistic action in  $\beta$ -cells in vivo, we observe that BZA acts as an ER $\alpha$  agonist with regard to the prevention of STZ-induced  $\beta$ -cell destruction. To our knowledge, BZA is the first SERM to show such beneficial action in female  $\beta$ -cells. This suggests that the combination CE/BZA is likely to exhibit beneficial actions with regard to  $\beta$ -cell survival from pro-apoptotic stresses in postmenopausal women.

## Conclusions

The combination CE/BZA and BZA alone prevent STZ-induced diabetes in female mice. This study demonstrates that BZA has no deleterious effect on  $\beta$ -cell survival in female mice and suggests that BZA acts as an ER $\alpha$  agonist with regard to female  $\beta$ -cells survival.

## Limitations

Although estrogens protection of mouse islets usually translates into human islets [2], the demonstration that BZA and the combination CE/BZA protect islet survival in women remains to be made.

## Additional Information

### Methods

#### Animals and surgery

8 week old female C57BL/6J mice (Jackson Laboratory, Bar Harbor, Maine) were housed with a 12 h light-dark cycle. The mice were subjected to either a bilateral OVX or a sham operation under anesthesia with 1.2% avertin solution (i.p.). After a recovery period of 2 weeks, mice were divided into 6 treatment groups as follows: 1) sham+vehicle, 2) sham+STZ+vehicle, 3) OVX+STZ+vehicle, 4) OVX+STZ+CE, 5) OVX+STZ+BZA, 6) OVX+STZ+CE+BZA. Diabetes was induced with 50 mg/kg STZ (dissolved in 50 mM citrate buffer, pH 4.5) or with citrate buffer alone injected intraperitoneally for 5 consecutive days. All compounds were administered orally by gavage to mice once daily for 5 weeks with vehicle (saline, 2% Tween 80, 0.5% methylcellulose), CE 2.5 mg/kg, BZA 3 mg/kg or CE 2.5 mg/kg +BZA 3 mg/kg in the vehicle solution. The administered dosages of CE and BZA were chosen to ensure optimal maintenance of the estrogen action in the absence of uterine growth [15] [16]. All mice received phytoestrogen-free HFD (TD04059, 52% Kcal from anhydrous milk fat, Harlan Teklad, Madison, WI, USA) and water ad libitum during the experimental period. At the end of the study, mice were euthanized by an overdose of avertin, and blood was collected by cardiac puncture. All animal work was performed in compliance with the Institutional Animal Care and Use Committee of Northwestern University and in accordance to NIH guidelines.

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#### Supplementary Material

Please see <https://sciencematters.io/articles/201605000017>.

#### Funding Statement

This study was funded by an investigator-initiated grant from Pfizer Inc and National Institutes of Health grant RO1DK074970. Pfizer had no involvement in the study design, the collection, analysis and interpretation of data. Pfizer had no involvement in the writing of the report and no involvement in the decision to submit the article for publication.

#### Ethics Statement

All animal work was performed in compliance with the Institutional Animal Care and Use Committee of Northwestern University and in accordance to NIH guidelines.

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